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Transglutaminase-mediated transamidation of serotonin, dopamine and noradrenaline to fibronectin: Evidence for a general mechanism of monoaminylation

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ABSTRACT

The activity of some small GTPases is regulated by covalent transamidation of serotonin (5-hydropxytryptamien) to glutamine residues of the enzymes. This process is mediated by transglutaminase (TGase) and is termed "serotonylation". In addition, serotonylation of neural proteins and proteins of the extracellular matrix such as fibronectin has been demonstrated. Here we show that the catecholamines dopamine (DA) and noradrenaline (NA) inhibit serotonylation of fibronectin and that DA and NA themselves can be selectively transamidated into fibronectin by TGase. All three biogenic monoamines also block TGase-mediated transamidation of another monoamine, monodansylacadaverine, into fibronectin, suggesting a general mechanism of TGase-mediated "monoaminylation".

1. Introduction

Outside the central nervous system (CNS) the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) plays important roles in the regulation of small GTPases and blood coagulation. In the latter process a subpopulation of platelets, so-called "coated-platelets", retain high levels of procoagulant proteins such as fibrinogen, von Willebrand factor, factor V and thrombospondin on the cell surface [1,2]. This process requires transglutaminase (TGase)-mediated transamidation of 5-HT into glutamine residues of specific acceptor proteins, a process which was termed "serotonylation". Tight interaction of serotonylated proteins with specific 5-HT binding sites on fibrinogen and thrombospondin then contribute to thrombus formation [3,4]. In addition, it has been shown, that serotonylation of small GTPases is involved in 5-HT release from blood platelets and insulin secretion from pancreatic beta-cells [5,6]. Very recently, serotonylation of vascular and neural proteins, as well as of C6 glioma cells and fibronectin has been described [7,8].

In the CNS besides 5-HT also the catecholamines dopamine (DA) and noradrenaline (NA) are important neurotransmitters which are implicated in the control of numerous behavioural and physiological functions. Having shown that TGase covalently transamidates [^{3}H]5-HT as well as the fluorescent amine monodansylcadaverine (MDC) into plasma fibronectin [7], in the current study we sought to determine whether DA and NA can block TGase-mediated serotonylation and whether TGase may covalently incorporate DA and NA into fibronectin. Therefore, we have compared the TGase-mediated transamidation of [^{3}H]5-HT, [^{3}H]DA and [^{3}H]NA into fibronectin to the one into bovine serum albumin. Moreover, we have analysed the inhibitory potencies of 5-HT, DA and NA on the TGase-mediated transamidation of MDC to fibronectin. In addition, we demonstrated the specificity of monoaminylation of fibronectin by identifying the MDC-labelled glutamine residues in fibronectin by mass spectrometry.

2. Materials and methods

2.1. Expression and purification of active recombinant TGase

For expression of the recombinant guinea pig TGase we applied a method using the chemical chaperone betaine as described [9]. TGase fraction was analysed on SDS–PAGE and Western blotting

Abbreviations: 5-HT, 5-hydroxytryptamine; DA, dopamine; MDC, monodansylcadaverine; NA, noradrenaline; TGase, transglutaminase

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using the antibody against TGase2 (Santa Cruz). Enzyme activity was determined as described [9,10].

2.2. Binding and transamidation of [^{3}H]5-HT into fibronectin

For the determination of binding and transamidation of tritiated monoamines, we used a modified ligand binding assay as described previously [7]. Ten micrograms of plasma fibronectin (Chemicon) was incubated in a final volume of 200 μL buffer (500 mM Tris-acetat, 8.75 mM $CaCl_2$ and 1.25 mM EDTA pH 7.5) with [^{3}H]5-HT, or [^{3}H]DA or [^{3}H]NA in the presence or absence of recombinant TGase and/or TGase inhibitors. After 3 h at room temperature the samples were rapidly filtered through Whatman GF/B glass fibre filters. The protein-bound radioactivity retained on the filters was determined using a Beckman LS60001IC scintillation counter. Specific transamidation of [^{3}H]monoamines by recombinant TGase was defined as the difference between total binding and transamidation to fibronectin in the presence of TGAse and simple binding of [^{3}H]monoamines to fibronectin in the absence of TGase.

To determine the inhibitory efficiencies of unlabelled 5-HT, DA and NA to block the specific transamidation of the radiolabelled monoamines, 10 μg of fibronectin was incubated with 250 nM [^{3}H]monoamine in presence and absence of recombinant TGase and with increasing concentrations of unlabelled 5-HT, DA and NA, respectively. Inhibition of specific transamidation of [^{3}H]monoamine to fibronectin was calculated as the difference between total binding/transamidation (in the presence of recombinant TGase) and binding without transamidation (in the absence of TGase) at the respective concentrations of unlabelled monoamines.

Data were analysed by non-linear regression analysis program (PRISM GraphPad), which fitted sigmoidal curves to the following equations:

$$V = V_{max}/(1 + [K_M/S]^n,$$

and

$$V/V_{max} = IC_{50}^n/(I^n + IC_{50}^n)$$

for competition experiments. V represents transamidation rate; V_{max}, maximal transamidation rate; S, substrate concentration; I, inhibitor concentration; IC_{50}, inhibitor concentration for half maximal transamidation inhibition; K_M, the Michaelis–Menten constant.

2.3. Electrophoresis and fluorography

Gel electrophoresis (SDS–PAGE) in 9% polyacrylamide gels was performed according to Laemmli [11]. For visualisation of TGase-mediated conjugation of MDC incorporation, 30 μg of fibronectin was incubated with 5 mM MDC as described above and subjected to SDS–PAGE. MDC labelled protein bands were visualised by fluorescence under UV-light.

2.4. Mass spectrometry

The major fluorescent band of MDC-labelled fibronectin (about 230 kDa) was excised from the gel and protein samples were in-gel digested with trypsin as described previously [12]. Extracted peptides were re-dissolved in 0.5 % trifluoroacetic acid and loaded on a C18 precolumn (Acclaim; Dionex, Idstein, Germany) using a RSLC-nano HPLC system (Dionex, Idstein, Germany). Peptides were then eluted with an aqueous-organic gradient, resolved on a C18 column (Acclaim; Dionex, Idstein, Germany) with a flow rate of 300 nl/min and electrosprayed into a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). A Triversa Automate (Advion biosciences, Ithaca, NY, USA) was used as ion source. Each scan cycle consisted of one FTMS full scan and up to seven ITMS dependent MS/MS scans of the seven most intense ions. Dynamic exclusion (30 s, mass width 20 ppm) and monoisotopic precursor selection were enabled. All analyses were performed in positive ion mode.

Extracted MS/MS spectra were searched against the Uniprot/Swissprot database (Mammalia) using the Phenyx search engine (GeneBio, Geneva, Switzerland) accepting common variable modifications and one missed tryptic cleavage. Peptide tolerance was ±10 ppm and MS/MS tolerance was ±0.6 Da. The MDC modification was manually defined and integrated in the databank searches.

2.5. Visualisation of transamidation of C6 cells by protein staining

Cells were plated into 10 cm dishes and grown up for 6 h in DMEM/F12 medium in the presence or absence of TGase (200 mU), DA (10 μM) or NA (10 μM). After overnight incubation, the cells were washed twice with PBS and incubated for 15 min at 37 °C with 4% paraformaldehyde (PFA). After 3 washes with PBS cells were incubated for 5 min at room temperature with Ponceau S solution (Fluka, 0.1% in 5% acetic acid) and washed three times with H_2O. Cell images were acquired on a Zeiss axiscope 2plus microscope (filter set 09; – BP 450–490, for fluorescence imagine) using an 20× planchromat lens. For quantification of protein expression, 20 identical quadrants each containing 10 cells were randomly picked and fluorescence was measured as the sum of intensity of all signals acquired with analySIS^B Software. Image recording and quantification of fluorescence were performed by independent operators. The mean intensity measured in the absence of TGase, DA and NA was taken as 100%. The calculation of the statistic standard deviations and *t*-tests were done with PRISM GraphPad.

3. Results

3.1. TGase-mediated transamidation of [^{3}H]5-HT to fibronectin and bovine serum albumin

First, we quantitatively characterised transamidation of [^{3}H]5-HT to fibronectin and bovine serum albumin(BSA) by recombinant TGase. To this end we purified recombinant guinea pig TGase as described in Section 2. In order to discriminate between pure binding of [^{3}H]5-HT to fibronectin and BSA and covalent transamidation by recombinant TGase we incubated 10 μg fibronectin (Fig. 1, bar A) or 100 μg BSA (Fig. 1, bar G) with 100 mU TGase and 250 nM [^{3}H]5-HT; here, radioactivity retained on the glass fibre filters reflects total binding and transamidation of [^{3}H]5HT by recombinant TGases. In order to determine 5-HT-displaceable binding/transamidation, we performed the same experiment in the presence of 500 μM 5-HT (Fig. 1, bars B and H). Incubation with [^{3}H]5-HT in the absence of recombinant TGase revealed total binding to fibronectin and BSA, respectively (Fig. 1, bars C and I), and the same experiment in the presence of 500 μM 5-HT revealed 5-HT-dispaceable binding to fibronectin (Fig. 1, bar D). Finally, incubation of fibronectin and BSA with [^{3}H]5-HT, recombinant TGase and 100 μM cystamine, which inhibits recombinant TGase, revealed total binding of [^{3}H]5HT to both proteins devoid of any [^{3}H]5-HT transamidation (Fig. 1, bars E and K). Calculating the difference between A, G (total binding and transamidation by recombinant TGase to fibronectin and BSA, respectively) and C, I (pure binding in the absence of TGase) reflects specific [^{3}H]5-HT transamidation by recombinant TGase. In three independent experiments 125.3 ± 4.8 pmol [^{3}H]5-HT were transamidated to 1 mg fibronectin. No TGase-mediated incorporation of [^{3}H]5-HT was observed into BSA indicating the specificity of fibronectin serotonylation. Saturation experiments revealed a maximal

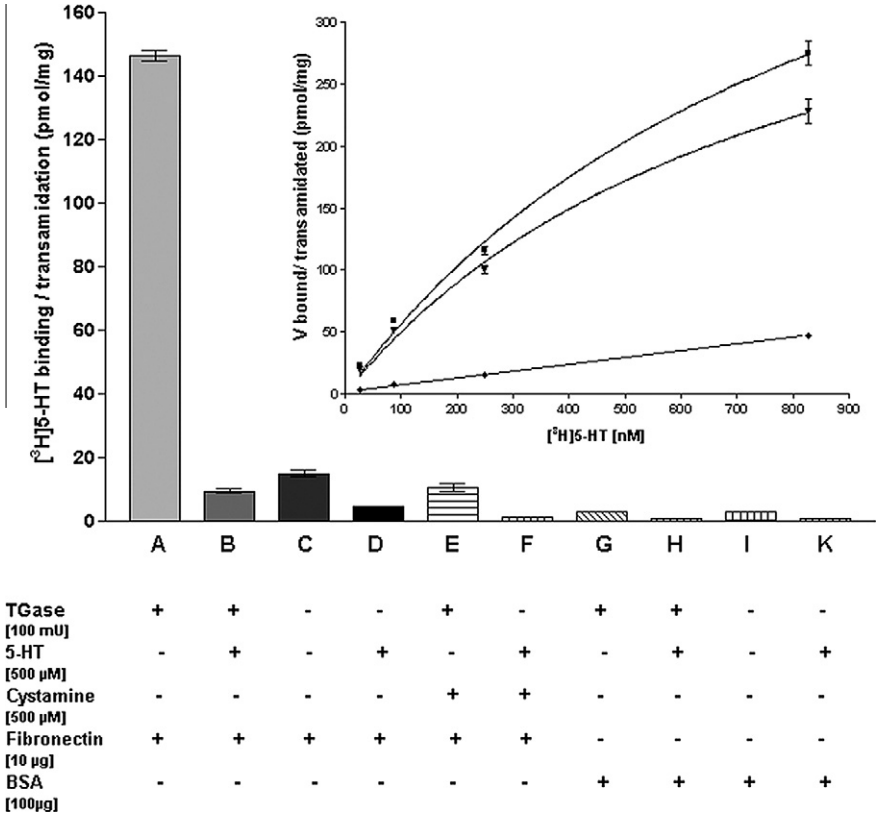

Fig. 1. Binding and transamidation of [^{3}H]5-HT to fibronectin and bovine serum albumin (BSA). 10 µg fibronectin (bars A–F) and 100 µg BSA (bars G–K) were incubated with 250 nM [^{3}H]5-HT and 100 mU TGase (bar A and G) for 3 h at room temperature as indicated. In bar B/H and D 500 µM unlabelled 5-HT was added to determine unspecific binding/transamidation. In bar E and K fibronectin and BSA were incubated with 500 µM cystamine to determine specific and unspecific binding by inhibition of TGase. Bar F presents, in presence of 500 µM 5-HT and cystamine, inhibition of specific and unspecific binding. Specific transamidation of [^{3}H]5-HT mediated by recombinant TGase2 calculated as the difference of A (=total binding and transamidation by recombinant TGase) and C (=total binding), was 123.1 pmol/mg in this representative experiment. The inset shows saturation of [^{3}H]5-HT binding (♦, corresponding to bar C), the sum of [^{3}H]5-HT binding and transamidation (■, corresponding to bar A) and specific transamidation as calculated from the difference between A and C-values (▼). The maximal incorporation for [^{3}H]5-HT into fibronectin in this representative experiment was 433.3 pmol/mg with a K_M = 677.3 nM. Error bars (shown when larger as the symbol used) represent standard error of the mean.

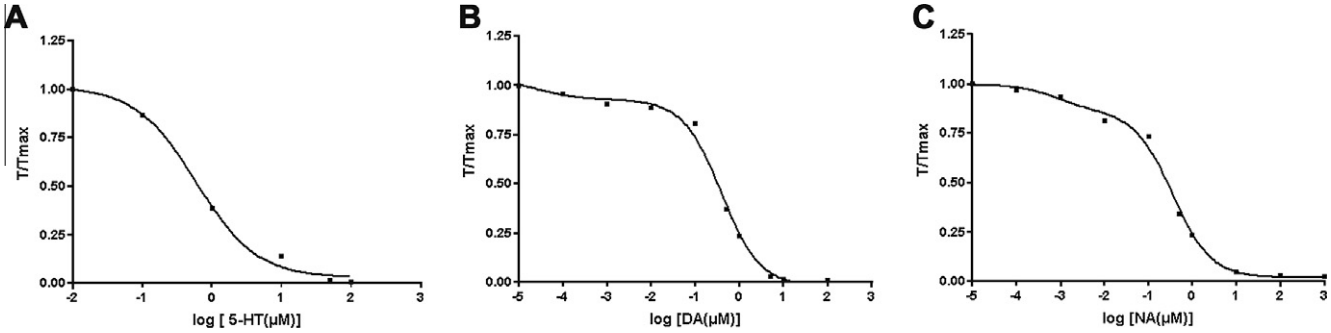

Fig. 2. Inhibition of [^{3}H5-HT transamidation to fibronectin. The IC_{50} values for the inhibition of [^{3}H]5-HT transamidation to fibronectin, obtained in these particular experiments were 549 nM for unlabelled 5-HT, 224 nM for DA and 256 nM for NA.

serotonylation of fibronectin of 424.6 ± 14.1 pmol/mg fibronectin with a K_M value of 794.1 ± 68.5 nM (n = 3).

3.2. Inhibition of [^{3}H]5-HT transamidation to fibronectin by 5-HT, DA and NA

Next we analysed whether transamidation of [^{3}H]5-HT to fibronectin is inhibited by unlabelled 5-HT, DA and NA as described in Section 2. As shown in Fig. 2, all three monoamine dose-dependently fully inhibited specific TGase-mediated incorporation of [^{3}H]5-HT into fibronectin. Non-linear regression analysis revealed IC50 values for 5-HT = 561 ± 19.3 nM (n = 3), DA = 235 ± 21.7 nM (n = 3) and NA = 259 ± 21.0 nM (n = 3). Representative experiments are shown in Fig. 2.

3.3. TGase-mediated transamidation of [^{3}H]DA to fibronectin and bovine serum albumin

Because DA dose-dependently inhibited specific transamidation of [^{3}H]5-HT into fibronectin, we next characterised TGase-mediated incorporation of [^{3}H]DA into fibronectin and BSA. As shown in Fig. 3, we performed the same set of experiments as described

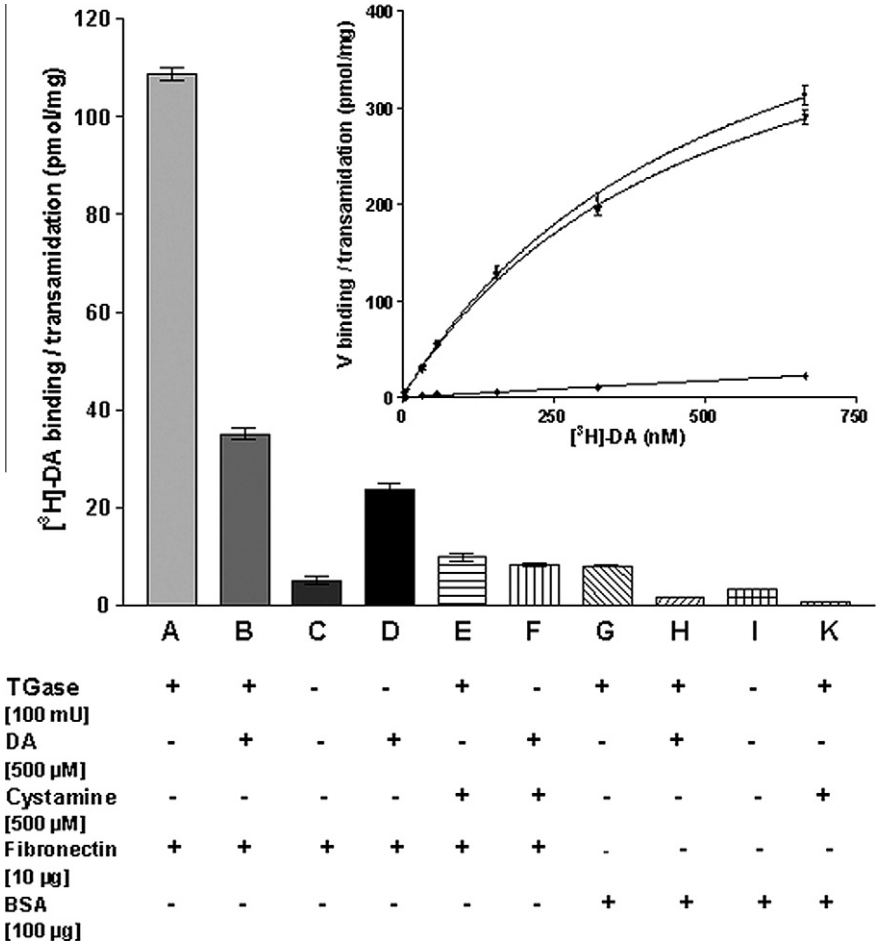

Fig. 3. Binding and transamidation of [^{3}H]DA to fibronectin. 10 µg fibronectin (bars A–F) and 100 µg bovine serum albumin (bars G–K) were incubated with 200 nM [^{3}H]DA and 100 mU TGase (bar A and G) for 3 h at room temperature as indicated. In bar B/H and D 500 µM unlabelled DA was added to determine unspecific binding/transamidation. In bar E and K fibronectin and bovine serum albumin were incubated with 500 µM cystamine to determine specific and unspecific binding by inhibition of exogenous TGase. Bar F presents, in presence of 500 µM DA and cystamine, inhibition of specific and unspecific binding. In this particular experiments transamidation of [^{3}H]DA mediated by recombinant TGase2 was calculated as the difference of A (total binding and transamidation by recombinant TGase) and C (=total binding) and reflected 102.3 pmol/mg. The inset shows saturation of [^{3}H]DA binding (♦, corresponding to bar C), the sum of [^{3}H]DA binding and transamidation (■, corresponding to bar A) and specific transamidation as calculated from the difference between A and C-values (▼). The maximal incorporation for [^{3}H]DA in the representative experiment was 391.5 pmol/mg with a K_M = 434.4 nM. Error bars are shown where larger than the symbol used and represent standard error of the mean.

above for the transamidation of [^{3}H]5-HT to both proteins and calculated the specific transamidation of [^{3}H]DA as the difference between A, G (binding and transamidation by recombinant TGase) and C, I (binding in the absence of TGase). In three independent experiments 103.8 ± 10.2 pmol [^{3}H]DA were transamidated to 1 mg fibronectin; no TGase-mediated incorporation of [^{3}H]DA was observed into BSA. Saturation experiments revealed a maximal incorporation of [^{3}H]DA into fibronectin of 431 ± 34.1 pmol/mg fibronectin with a K_M value of 448.2 ± 14.2 nM (n = 4).

3.4. Inhibition of [^{3}H]DA transamidation to fibronectin by 5-HT, DA and NA

Next we determined the potency of unlabelled 5-HT, DA and NA to inhibit the TGase-mediated incorporation of [^{3}H]DA to fibronectin. As shown in Fig. 4, only 5-HT and NA completely inhibited specific TGase-mediated incorporation of [^{3}H]DA into fibronectin in a dose-dependent manner whereas DA maximally inhibited 89.7 ± 1.1% of [^{3}H]DA incorporation (n = 3). Non-linear regression analysis revealed IC50 values for 5-HT = 2.2 ± 0.04 mM (n = 3), DA = 989.4 ± 20.1 nM (n = 3) and NA = 50.6 ± 0.7 µM (n = 3). Representative experiments are shown in Fig. 4.

3.5. TGase-mediated transamidation of [^{3}H]NA to fibronectin and bovine serum albumin

Next we next characterised TGase-mediated incorporation of [^{3}H]NA into fibronectin and BSA. As shown in Fig. 5, we performed the same set of experiments as described above for the transamidation of [^{3}H]5-HT and [^{3}H]DA to both proteins and calculated the specific transamidation of [^{3}H]NA as the difference between A, G (total binding and transamidation by recombinant TGase) and C, I (total binding in the absence of TGase). In three independent experiments 45.8 ± 3.3 pmol [^{3}H]NA were transamidated to 1 mg fibronectin, whereas no TGase-mediated incorporation of [^{3}H]NA was observed into BSA.

Saturation experiments revealed a maximal incorporation of [^{3}H]NA into fibronectin of 154 ± 20.4 pmol/mg fibronectin with a K_M value of 441.5 ± 12.3 nM (n = 4).

3.6. Inhibition of [^{3}H]NA transamidation to fibronectin by 5-HT, DA and NA

As shown in Fig. 6, 5-HT and NA dose-dependently inhibited specific TGase-mediated incorporation of [^{3}H]NA into fibronectin

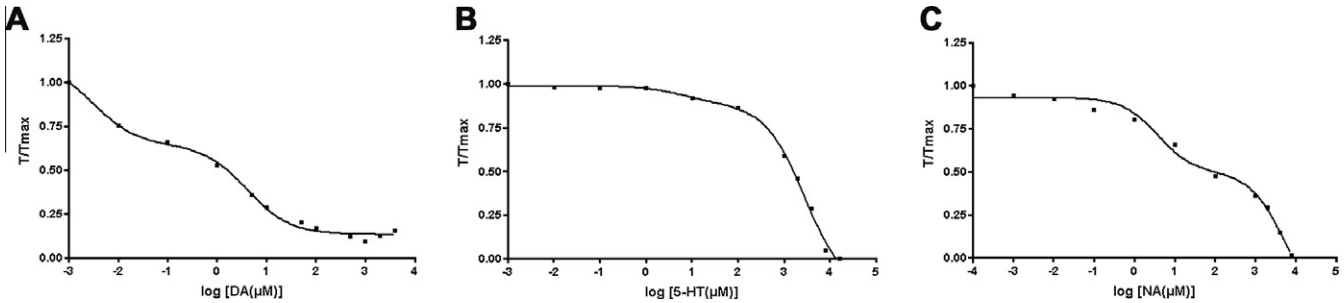

Fig. 4. Inhibition of [^{3}H]DA transamidation to fibronectin. The IC_{50} values for the inhibition of [^{3}H]DA transamidation to fibronectin, obtained in this particular experiment was 953 nM for unlabelled DA, 2.3 mM for 5-HT, and 51.5 μM for NA.

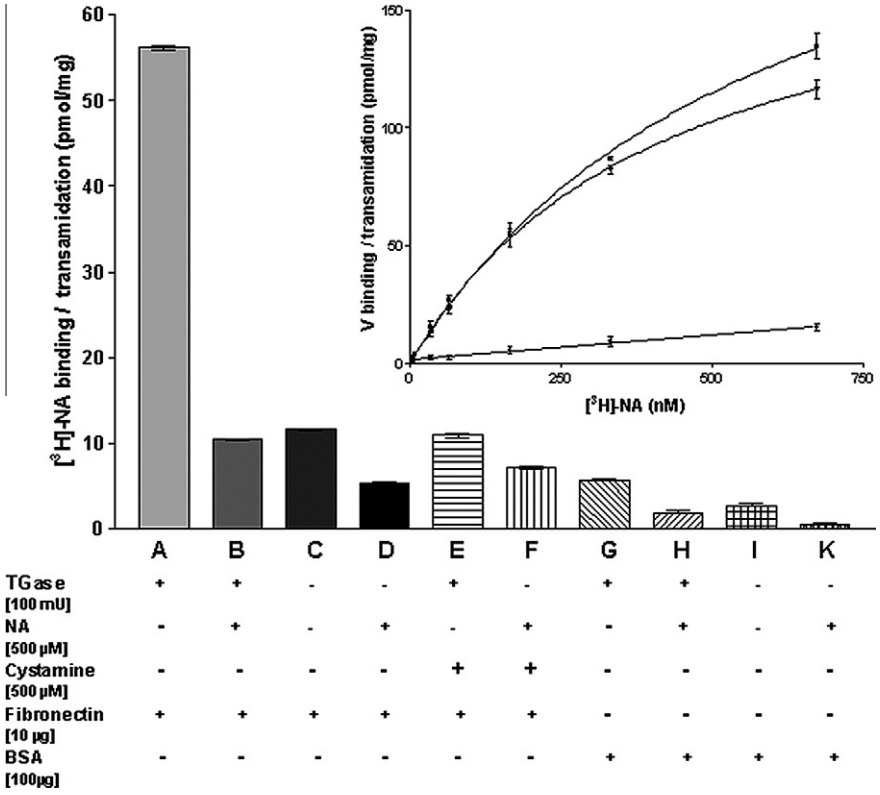

Fig. 5. Binding and transamidation of [^{3}H]NA to fibronectin. 10 μg fibronectin (bar A-F) and 100 μg bovine serum albumin (G-K) were incubated with 200 nM [^{3}H]NA and 100 mU TGase (bar A and G) for 3 h at room temperature as indicated. In bars B/H and D 500 μM unlabelled NA was added to determine unspecific binding/transamidation. In bars E and K fibronectin and bovine serum albumin were incubated with 500 μM cystamine to inhibit exogenous TGase activity. Bar F presents, in presence of 500 μM NA and cystamine, inhibition of specific and unspecific binding. In this particular experiments transamidation of [^{3}H]NA mediated by recombinant TGase2 was calculated as the difference of A (total binding and transamidation by recombinant TGase) and C (total binding without transamidation) and reflected 44.6 pmol/mg. The inset shows saturation of [^{3}H]DA binding (♦, corresponding to bar C), the sum of [^{3}H]DA binding and transamidation (■, corresponding to bar A) and specific transamidation as calculated from the difference between A and C-values (▼). The maximal incorporation for [^{3}H]NA in the representative experiments was 189.9 pmol/mg with a K_M = 426.3 nM. Error bars are shown where larger than the symbols used and represent standard error of the mean.

with IC50 values for 5-HT of 126.1 ± 3.1 μM (n = 3), and NA of 1.1 ± 0.03 μM (n = 3). DA, however, only b maximally 77.2 ± 1.8% of TGase-mediated [^{3}H]NA incorporation with an IC 50 value of 2.1 ± 0.05 μM (n = 3).

3.7. Inhibition of TGase-mediated incorporation of MDC into fibronectin by 5-HT, DA and NA

Recently we had shown that TGase covalently transamidates the fluorescent monoamine MDC into fibronectin. After subjection to SDS–PAGE MDC-labelled fibronectin could be visualised by UV illumination. MDC-labelling was inhibited by cystamine and dose-dependently reduced in the presence of 5-HT [7]. Here, we compared the potencies of 5-HT, DA and NA to inhibit TGase-mediated transamidation of MDC to fibronectin. As shown in Fig. 7, MDC-labelling was partly diminished in the presence of 40 mM 5-HT, more strongly diminished in the presence of 40 mM NA, and totally blocked by 10 mM DA.

3.8. Identification of MDC-labelled glutamine residues in fibronectin

In order to identify MDC-labelled glutamine (Gln; Q) residues of fibronectin after SDS–PAGE we excised fluorescent bands of about 230 kDa, trypsinized the protein in-gel and performed mass spectrometry (MS) as described in Section 2. As shown in Table 1, eleven MDC-labelled Gln residues were identified.

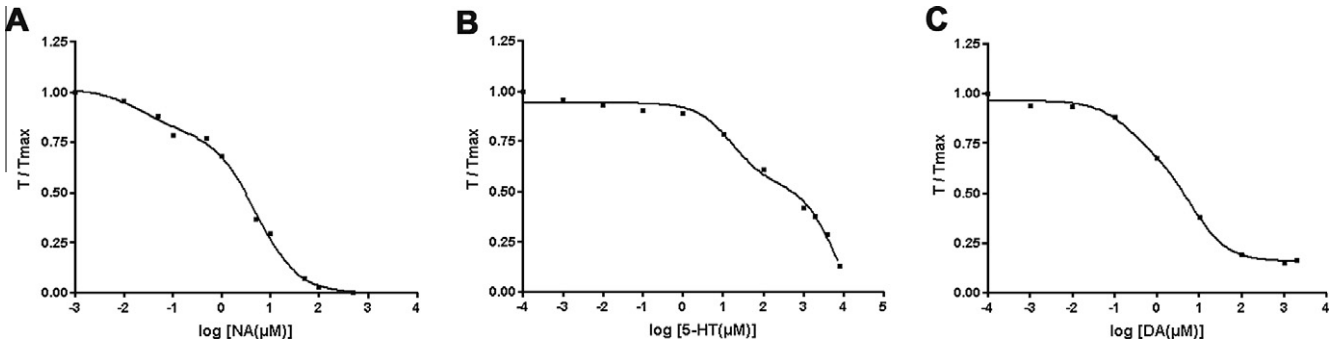

Fig. 6. Inhibition of [^{3}HNA transamidation to fibronectin. The IC_{50} values for the inhibition of [^{3}H]NA transamidation to fibronectin, obtained in this particular experiments were 1.0 μM for unlabelled NA, 124 μM for 5-HT, and 2.3 μM for DA.

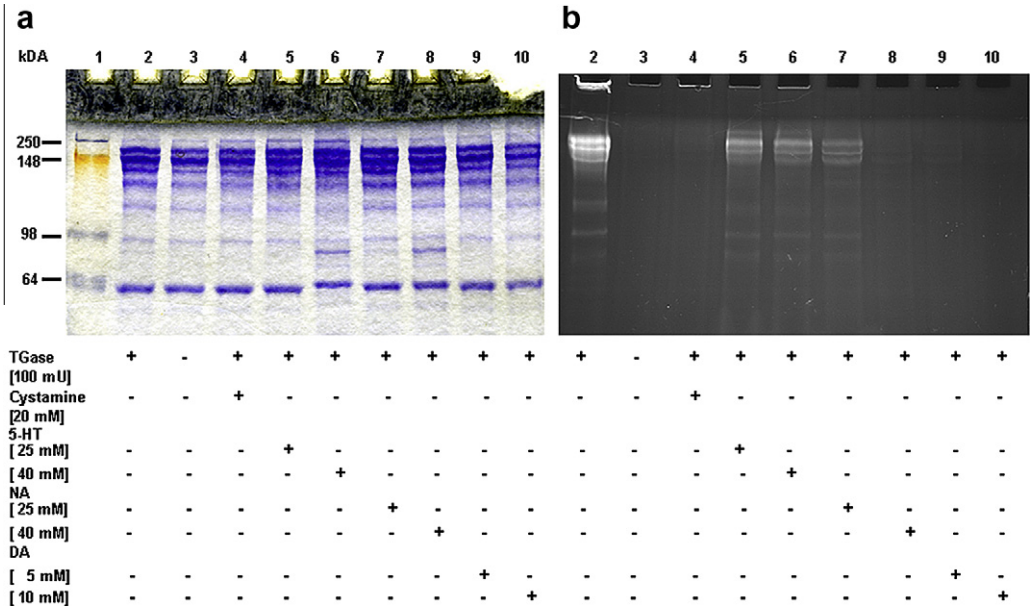

Fig. 7. TGase mediated incorporation of MDC into human plasma fibronectin. 30 μg fibronectin were incubated with 5 mM MDC in the presence of 200 mU recombinant TGase, cystamine, 5-HT, DA and NA as indicated and as described in Section 2. (a) Coomassie stain of assay panel (b), fluorescence of MDC incorporation.

Table 1
Modified amino acid residues are bold and underscored; *z* charge; *m*/*z* mass-over-charge; AA amino acid. The AA-position refers to fibronectin (P02751). Peptides were identified with Phenyx (BeneBio). Modified Glutamine residues in fibronectin.

#	Amino acid sequence	*z*	*m*/*z*	AA-Position	Modification
1	K/HYQINQ**Q**WER/T	3	573.94	27–36	1 MDC
2	R/HTSV**Q**TTSSGSGPFTDVR/A	3	728.013	273–290	1 MDC
	R/HTSV**Q**TTSSGSGPFTDVR/A	2	1091.515	273–290	1 MDC
3	K/GLKPGVVYEGQLISIQ**Q**YGHQEVTR/F	3	1039.88	670–694	1 MDC
4	R/WSRP**Q**APITGYR/I	3	583.968	831–842	1 MDC
5	K/LGVRPS**Q**GGEAPR/E	3	547.956	1117–1129	1 MDC
6	R/TEIDKPS**Q**MQVTDVQDNSISVK/W	3	927.454	1540–1561	1 MDC
	R/TEIDKPS**QM**QVTDVQDNSISVK/W	3	932.786	1540–1561	1 MDC, 1 Oxidation_M
	R/TEIDKPS**Q**M**Q**VTDVQDNSISVK/W	3	1033.501	1540–1561	2 MDC
	R/TEIDKPS**Q**M**Q**VTDV**Q**DNSISVK/W	3	1139.547	1540–1561	3 MDC
7	R/RPGGEPSPEGTTG**Q**SYNQYSQR/Y	3	905.413	2125–2146	1 MDC
	R/RPGGEPSPEGTTG**Q**SYNQYS**Q**R/Y	3	1011.463	2125–2146	2 MDC
	R/RPGGEPSPEGTTG**Q**SYN**Q**YS**Q**R/Y	3	1117.509	2125–2146	3 MDC

3.9. Transamidation of DA and NA into living C6-glioma cells

Recently we had shown that TGase-mediated transamidation of serotonin to living C6 glioma cells induced an aggregation of extracellular protein matrices adjacent to and between single cells [7]. Consequently, we now studied whether "dopaminylation" and noradrenylation" of C6 glioma cells also affects extracellular protein expression. To this intent we incubated C6 cells with 10 μM DA or 10 μM NA in the absence or presence of TGase and visualised protein expression by staining with Ponceau S as described in Section 2. Bright-field microscopy revealed that individual cell morphology was not affected by TGase treatment. Protein staining

with Ponceau S, revealed more intense protein expression on and between TGase-treated C6 cells in the presence of DA or NA as compared to untreated cells (Fig. 8A and B). In order to compare protein expression between TGase-treated versus untreated cells more accurately, Ponceau S staining was excited at 450 nM (Fig. 8C) and the resulting fluorescence was quantified as described in Section 2. As shown in Fig. 8D, total protein expression on and between C6 cells quantified by Ponceau staining was augmented to 180.5 ± 5.8% by the addition of 10 μM DA and 180.7 ± 5.5% by the addition of 10 μM Na in the presence of 200 mU TGase (mean out of four independent experiments incl. S.E.M. values).

4. Discussion

The biogenic monoamine 5-HT had been shown to be transamidated inside cells to small GTPases rendering these proteins active [5,6]. This process is mediated by TGases and has been termed "serotonylation". Recently also the specific serotonylation of fibro-

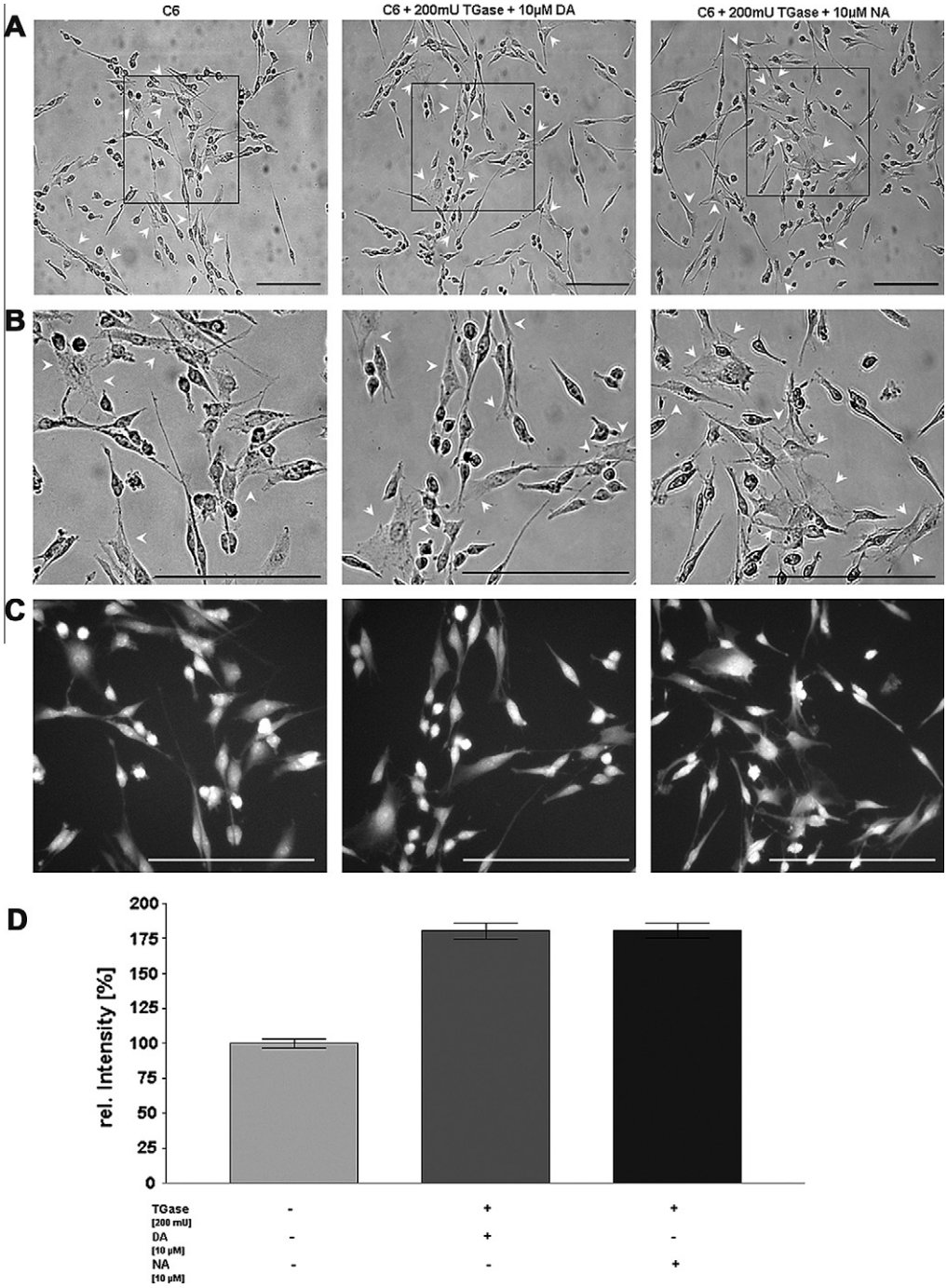

Fig. 8. TGase-mediated transamidation of DA and NA into living C6 glioma cells. Cells were incubated in the absence of 200 mU TGase, 10 μM DA or 10 μM NA as indicated on the top of the figure. Protein staining with Ponceau S; arrow heads indicate more pronounced extracellular protein accumulation between and adjacent to monoaminylated C6 cells as compared to not-monoaminylated cells. B, represents a zoom from A. C, fluorescence of Ponceau staining upon excitation at 450 nm; D, quantitative evaluation of Ponceau fluorescence revealed enhanced protein staining to 180.5 ± 5.8% by the addition of 10 μM DA and 180.7 ± 5.5% by the addition of 10 μM NA in the presence of 200 mU TGase as compared to non-treated cells (100 ± 3.2%). The data are the mean out of four independent experiments incl. S.E.M. values. Bars represent 100 μm.

nectin, a protein of the extracellular matrix, has been reported [7,8]. In the latter study it had been shown, that TGase also can selectively transamidate the fluorescent monoamine MDC to fibronectin and that this transamidation was reduced by 5-HT. The first major finding of our present study is that TGase-mediated transamidation of [^{3}H]5-HT and MDC into fibronectin is blocked by DA and NA. Moreover, similarly to our findings for [^{3}H]5-HT [7], TGase also covalently incorporated [^{3}H]DA and [^{3}H]NA into fibronectin, but not into BSA. Saturation experiments revealed, that the maximal incorporation rate of all three monoamines did not differ significantly (5-HT: 425 ± 60; DA 431 ± 14 and NA: 441 ± 61 pmol/mg fibronectin), whereas the K_M values for transamidation by TGase varied from 154 nM for [^{3}H]NA over 448 nM for DA to 749 nM for 5-HT. These findings suggest that TGase transamidates the same amount of glutamine residues on fibronectin with 5-HT, DA and NA but with different affinities. This is also reflected by the results of our inhibition experiments where the three monoamines blocked the transamidation of radiolabelled monoamines with substantial different efficiencies. At this point it should be noted that with the exception of the inhibition of transamidation of [^{3}H]5-HT by unlabelled 5-HT none of the other displacement profiles followed an adequate non-linear regression fit. The unusual inhibition profiles observed in the displacement studies were reproducible in each single experiment.

Monoamine incorporation into glutamine residues is known to take place at a thioester-intermediate formation between a cysteine residue of the TGase and the glutamine residue of the donor protein [13,14]. Thus our heterogeneous displacement curves suggested multiple glutamine residues in fibronectin as target for transamidation which are differentially accessible to 5-HT, DA and NA. In order to determine possible target glutamine residues for monoaminylation we isolated the fluorescent MDC-labelled fibronectin band at 230 kDa after SDS–PAGE and identified MDC-modified glutamine residues by mass spectrometry. Using this approach we found eleven glutamine residues which were modified by MDC. The differential accessibility of these residues to the different monoamines is reflected by the finding that MDC-transamidation is most effective blocked by DA > NA > 5-HT (see Fig. 7).

In addition, transamidation of living C6 glioma cells with DA and NA by recombinant exogenous TGase led to a profound protein aggregation adjacent to and between single cells without affecting the cell morphology. Here, the extent of extracellular protein aggregation upon "dopaminylation" and "noradrenylation" was comparable to the one which we had measured upon serotonylation of C6 cells in a recent study [7]. These findings suggest a common effect of TGase-mediated "monoaminylation" of extracellular proteins.

In all our experiments we have used recombinant TGase2, because this enzyme is the major TGase isoform in the brain where it is expressed both intra- and extra-cellular [13,14]. TGase2 had also been shown to serotonylate proteins of smooth muscle cells [8]. Very recently it has been shown in TGase2-knock-out mice that TGase2-independent TGase activity exists in the vasculature and that aortic smooth muscle cells also express TGase 1 and TGase4 in addition to TGase2 [15]. These findings raise the question whether other TGase isoforms may also catalyse a general mechanism of monoaminylation comparable to TGase2.

In summary, our findings on TGase-mediated transamidation of the monoamines 5-HT, DA, NA and MDC into fibronectin suggest a more general mechanism of "monoaminlyation" beyond the already described mechanism of serotonylation. Comparable to our findings here also the transamidation of NA to proteins of vascular smooth muscle cells has been reported [16] and a new role for biogenic monoamines has also been recently discussed in theory with respect to the regulation of small GTPases, G-proteins, lipid signalling and metabolic enzymes [17]. Here we present the first compelling biochemical evidence to support this hypothesis and hope that these findings will spur further studies on the identification of other specific target proteins for monoaminylation and on the physiological role of monoaminylation.

Conflict of interest

The authors declare no conflict of interest.

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